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# Cytochrome P450 expression and activities in human tongue cells and their modulation by green tea extract

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## Abstract:

The expression, inducibility, and activities of several cytochrome P450 (CYP) enzymes were investigated in a human tongue carcinoma cell model, CAL 27, and compared with the human liver model HepG2 cells. The modulation effects of green tea on various CYP isoforms in both cell lines were also examined. RT-PCR analysis of CAL 27 cells demonstrated constitutive expression of mRNA for CYPs 1A1, 1A2, 2C, 2E1, 2D6, and 4F3. The results were negative for CYP2A6, 2B6/7, 3A3/4, and 3A7. Both cell lines displayed identical expression and induction profiles for all of the isoforms examined in this study except 3A7 and 2B6/7, which were produced constitutively in HepG2 but not Cal-27 cells. CYP1A1 and 1A2 were both induced by treatment with β-napthoflavone as indicated by RT-PCR and Western blotting, while CYP2C mRNA was upregulated by all-trans retinoic acid and farnesol. RT-PCR and Western blot analysis showed that the expressions of CYP1A1 and 1A2 were induced by green tea extract (GTE), which also caused an increase in mRNA for CYP2E1, CYP2D6, and CYP2C isoforms. The four tea catechins, EGC, EC, EGCG and ECG, applied to either HepG2 or Cal-27 cells at the concentration found in GTE failed to induce CYP1A1 or CYP1A2, as determined by RT-PCR. Of the isoforms that were apparently induced by GTE, only 7-ethoxycoumarin deethylase (ECOD) activity could be detected in CAL 27 or HepG2 cells. Interestingly, mRNA and protein for CYP1A1 and CYP1A2 were detected in both cell lines, and although protein and mRNA levels of CYP1A1 and CYP1A2 were increased by GTE, the observed ECOD activity in both cell lines was decreased.

## Keywords:

Cytochrome P450; Tongue; Oral; Green tea; RT-PCR

## Article:

### INTRODUCTION

The major site for production of cytochrome P450 (CYP) enzymes is the liver, although various forms of cytochrome P450 are expressed in other organs and tissues at significant levels (Lechevrel et al., 1999). The extrahepatic expression of CYPs is not as well characterized as it is in the liver, and in particular, there are very few studies that have addressed CYP expression in oral tissue. CYP expression in the human tongue has never been reported. However, constituents present in food and beverages, chemicals in tobacco products, and many drugs are candidates for oral absorption and oxidative metabolism by the CYP system. Moreover, given the tremendous potential for foreign chemical exposure, alteration of CYPs in the oral cavity may play an important role in the activation of carcinogens or has influence on drugs prescribed for oral or sublingual administration. Several observations have been made that suggest metabolism by CYPs in the oral epithelium does occur. For example, cultured human oral keratinocytes were found to metabolize benzo[a]pyrene to DNA-binding species (Autrup et al., 1985) and exposure to N-nitrosamines and polycyclic aromatic hydrocarbons (PAHs) was shown to induce oral squamous cell carcinoma in rats (Rivenson et al., 1988). Furthermore, the risk of developing oral

tumors has been associated with tobacco use and excessive alcohol consumption (Yamazaki et al., 1992), which could result from an increase in metabolic activation of tobacco-specific nitrosamines by the ethanol- inducible CYP2E1 (Perrot et al., 1989).

CYP1A1 and 1A2 are also responsible for the metabolism of many aromatic compounds found in the human environment and diet, and have received much attention. Both of these isoforms can be induced by a variety of xenobiotics, including 2,3,7,8,-tetrachlorodibenzo-p-dioxin (TCDD) and β-napthoflavone (Ghosh et al., 2001) in liver and extrahepatic tissue. In addition to xenobiotics, a variety of endogenous compounds have been shown to regulate CYP expression. For example, CYP2C7 is known to be regulated by all-trans retinoic acid through the retinoic acid receptor (RAR) and retinoid X receptor (RXR) (Westin et al., 1997). In the current study, the ability of β-napthoflavone and retinoic acid to induce their respective P450 isoforms in cultured human tongue carcinoma cells was evaluated.

In recent years, green tea has received a great deal of attention regarding its anti-oxidant and anti-cancer activities. Although the mechanism underlying these effects is not yet fully understood, many studies have shown that the induction or inhibition of cytochrome CYPs by tea components may have a significant contribution. It is thought that tea and its polyphenols may inhibit carcinogenesis by blocking the metabolic activation of N-nitroso compounds to potentially toxic, mutagenic, and carcinogenic intermediates by various forms of CYPs (Shu and Hollenberg, 1996). It was reported that the effect of green tea and its polyphenols on CYP1A was complex. For example, several studies using rats as models have shown significant increases in the liver CYP1A1 and 1A2 proteins after rats were given 2–3% (w/v) aqueous extract of green tea (Maliakal et al., 2001). However, green tea was found to markedly inhibit demethylation of ethoxyresorufin and methoxyresorufin, reactions carried out by the CYP1A subfamily (Bu-Abbas et al., 1994). Moreover, a study by Muto et al. (2001) demonstrated the nonspecific inhibitory effects of tea polyphenols toward several human CYPs. These studies suggest that inhibition of specific CYPs by green tea may be one of the protection mechanisms against genotoxicity (Edenharder et al., 2002) and could also lead to significant pharmacological interactions. Although inhibition of P450 activity may be related to polyphenolic compounds in the tea, Chen et al. (1996) have shown that in rats, the induction of CYP1A2 is most likely due to caffeine present in the extract.

Our previous studies using animal models (rat, rabbit, and bovine) established the expression of specific CYPs and CYP-dependent activities in animal tongues (Yang et al., 2003). In the present study, RT-PCR analysis was conducted to examine the constitutive expression of 10 CYPs in a human tongue cell line, CAL 27, and the inducibility of specific CYPs in response to different chemical inducers. In addition, the effects of commercially available green tea extract (GTE) on oral CYP expression and activities were examined. Finally, the parallel assessments of tongue and liver cells (HepG2) provide a comparison between the tongue carcinoma cell line and a commonly used human hepatoma model cell line. The results extend our knowledge of CYP expression, induction, and activities to cultured human tongue cells and provide fundamental information concerning dietary interactions in the tongue that may be of pharmacological or toxicological significance.

## MATERIALS AND METHODS

### *Cell culture.*

The CAL 27 human tongue squamous cell line was purchased from American Type Culture Collection (ATCC, Rockville, MD). The HepG2 human liver cell line was kindly provided by Dr. George Loo (The University of North Carolina at Greensboro, NC). CAL 27 cells were maintained as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1x pen-strep (PS). HepG2 cells were grown in the same medium mentioned above and supplemented with the same essential ingredients used for CAL 27 cells.

### *Chemical inducer and green tea extract treatment.*

Chemical inducers (β-napthoflavone, all-trans retinoic acid, farnesol, EGC, EGCG, EC, and ECG) were all purchased from Sigma (St. Louis, MO). Green tea extract (standardized to contain 50% polyphenols) was

manufactured by Spring Valley (Bohemia, NY) and purchased from a local store. Both HepG2 and CAL 27 cells were maintained in 75 $cm^2$ tissue culture flasks. After the cells reached about 80% confluency, they were trypsinized and harvested. An equal number of cells (1.5 x $10^6$ cells/10 ml media) was transferred to each experimental 25 $cm^2$ flask to be treated the next day. Cells were treated with the chemical inducers for 1 and 5 h and with green tea extract (GTE) for 6 h. After treatment, cells were collected and were subjected to total RNA isolation according to the protocol provided with the RNA isolation kit described below.

All potential chemical inducers, including the four catechins and GTE, were dissolved in DMSO. Each inducer was added to the respective cell culture media to reach the final concentration of β-napthoflavone (100 µM β-NF), all-trans retinoic acid (20 µM RA) (Westin et al., 1997), and farnesol (80 µM) (Howard et al., 2000). GTE stock solution was added to the respective cell culture media to reach the final concentration of 25, 50, 75, and 100 µg/ml (Williams et al., 2000). The vehicle (DMSO)-treated cells served as the control group for each experiment. HepG2 liver cells were used for the positive control and for comparison purposes in this study. For each condition, two independent experiments were performed in duplicates.

### *HPLC analysis of catechins in GTE.*

Standard curves for EGC (0.05–0.20 mg/ml), EC (0.01–0.04 mg/ml), EGCG (0.1–0.2 mg/ml), and ECG (0.01–0.04 mg/ml), all in DMSO, were generated by the method of Lee et al. (2000) using a Haisil-100 C18 HPLC column (150 x 4.6 mm, particle size of 5 µm) and a Shimadzu HPLC system consisting of an LC-10AL dual pumping unit, an SCL-10A controller, an FC10AL quaternary solvent mixer, a DGU14A degasser, an SPD-M10A diode array detector, and a SIL-10A auto-sampling unit. A standard GTE solution in DMSO (25 mg/ml) was also analyzed using the same method, and the four individual catechins were quantified in this extract using the standard curve data.

### *Total RNA isolation and RT-PCR analysis.*

The SV Total RNA Isolation System protocol (Promega Co., Madison, WI) was used for isolation of total RNA. The manufacture protocol involved disruption of cells, denaturation of nucleoprotein complexes, inactivation of endogenous ribonuclease (RNase) activity, and finally removal of proteins and DNA. Reverse transcription of 10 µg of RNA to a corresponding amount of cDNA was carried out using a First-Strand cDNA Synthesis Kit protocol (Amersham Biosciences Co., Piscataway, NJ).

The PCR reactions of single-stranded cDNA from 10 µg total RNA were performed in a final volume of 50 µl consisting of 5 µl cDNA, 5 µl 10 x PCR buffer, 200 µM of each deoxynucleotide triphosphate, 2.5 mM $MgCl_2$, 1.5 U Taq DNA polymerase (Promega Co.), and 50 pmol each of the forward and reverse primer. The reaction mixtures were heated at 95 °C for 4 min and then cycled 30 times through a 1 min denaturation step at 94 °C, a 1 min annealing step at 60 °C, and a 2 min extension step at 72 °C in a DNA Thermal Cycler apparatus purchased from Perkin-Elmer Co. (Wellesley, MA). A 4.0 min extension time at 75 °C was included at the end of 30 cycles, and this was followed by incubation at 4 °C for an indefinite period. These conditions were used for the amplifications of β-actin and all CYP isoforms except for 3A3/4, 3A7, and 4F3, where the annealing step was performed at 52 °C. All reactions were conducted with β-actin primers as internal controls. Isolated RNA from human HepG2 cells was used as a positive control in each RT-PCR experiment for the respective P450 isoforms. An aliquot of 20 µl from each reaction was separated on a 2% agarose gel (Sigma) and visualized by ethidium bromide staining. The bands were quantified by densitometry using Kodak ID Image Analysis Software. All PCR products were of the correct size ensuring that the results reflected amplification of cDNA and not genomic DNA. The primer sequences for RT-PCR involving 1A1, 1A2, 2A6, 2B6/7, 2C, 2D6, and 2E1 were taken from the literature (Vondracek et al., 2001), as were those for 3A3/4, 3A7 (Hakkola et al., 1996), and 4F3 (Christmas et al., 2001). All primers were synthesized by Integrated DNA Technologies (Coralville, IA). Primer sequences, expected PCR fragment length, and annealing temperatures for the isoforms examined are listed in Table 1.

### *Preparation of S9 and microsomal fraction.*

S9 fractions were prepared according to a protocol from Gentest (Woburn, MA). Briefly, both CAL 27 and HepG2 cells (approximately 80% confluent) cultured in 75 cm2 cell culture flasks with 6 x $10^6$ cells/flask were treated with 100 µM β-NF for 1 and 5 h or GTE (100 µg/ml) for 6 h. After treatment, old medium was removed and 1.5 ml new medium was added to each 75 $cm^2$ flask. The cells were scraped out using a rubber policeman and transferred to three 1.5 ml Eppendorf tubes in a total volume of 0.5 ml in each tube. The same procedures were repeated for each flask so the total volume in each tube was 1 ml. The cells were centrifuged at 5000–8000 x g for 5 min and the pellet was collected and resuspended with 100 µl 10 mM tris-acetate buffer (pH 7.4). The cells were then sonicated on ice at power level 1 for 30 s using a Sonic Dismembrator (Fisher Scientific). This was repeated a second time after a min interval. The cells were next centrifuged at 10 000 x g for 20 min and the supernatant (S9 fractions) was collected. The supernatant was subjected to centrifugation at 500000 x g for 30 min at 4 °C (Williams et al., 2000). The microsomal pellet was resuspended in the above buffer and protein concentrations were determined by the Lowry assay, using bovine serum albumin (BSA) as the standard protein. The isolated microsomal protein was then subjected to Western blot analysis.

Table 1
Primer sequences, expected PCR product sizes, and annealing temperatures for RT-PCR primers used for human CYP isoforms in this study

| Primer | Sequence | Product (bp) | Annealing temperature (°C) |
|---|---|---|---|
| β-actin | 5′ GGTCACAACTGCCATCTCG 3′; 5′ GTTCTGCCACTGGTTCACG 3′ | 202 | 60 |
| 1A1 | 5′ GGAGGCCTTCATCCTGGAGA 3′; 5′ CCTCCCAGCGGGCAACGGTC 3′ | 295 | 60 |
| 1A2 | 5′ GGAGGCCTTCATCCTGGAGA 3′; 5′ TCTCCCACTTGGCCAGGACT 3′ | 299 | 60 |
| 2A6 | 5′ CAACCAGCGCACGCTGGATC 3′; 5′ CCAGCATAGGGTACACTTCG 3′ | 423 | 60 |
| 2B6/7 | 5′ ACACAGTGAATTCAGCCACC 3′; 5′ TGGTGTGTTGGGTGACAATG 3′ | 289 | 60 |
| 2C | 5′ CCAGAGGTCACAGCTAAAGT 3′; 5′ CCTGCTGAGAAAGGCATGAA 3′ | 344 | 60 |
| 2D6 | 5′ CCTGCGCATAGTGGTGGCTG 3′; 5′ GCTTCTCCCAGACGGCCTCA 3′ | 353 | 60 |
| 2E1 | 5′ TGCCATCAAGGATAGGCAAG 3′; 5′ AATGCTGCAAAATGGCACAC 3′ | 356 | 60 |
| 3A3/4 | 5′ CCAAGCTATGCTCTTCACCG 3′; 5′ TCAGGCTCCACTTACGGTGC 3′ | 323 | 52 |
| 3A7 | 5′ CTATGATACTGTGCTACAGT 3′; 5′ TCAGGCTCCACTTACGGTCT 3′ | 474 | 52 |
| 4F3 | 5′ GAAACGGAATTGGTTCTTGG 3′; 5′ TGCATGATGTTCACACTC 3′ | 359 | 52 |

### *Western blot analysis.*

Microsomal proteins (100 µg total protein/lane) were resolved by denaturing electrophoresis on 10% polyacrylamide slab gels, sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) according to manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA). The rabbit anti-actin primary antibody and the goat anti-rabbit Ig were purchased from Sigma. The goat anti-rat primary polyclonal antibodies of CYP1A1/1A2 and CYP2E1 were purchased from Gentest. The immunocomplexes were developed using an enhanced chemiluminescent reagent (SuperArray, Bethesda, MD) and were compared to prestained protein standards (Bio-Rad Laboratories). The blots were then visualized using a CCD camera system, Image Station 440 CF (Kodak Digital Science). The bands were quantified by densitometry using Kodak ID Image Analysis Software.

### *Metabolism of various CYP substrates.*

The substrates, 7- methoxy-4-(aminomethyl)-coumarin (MAMC), and the reaction products, 4-hydroxydiclofenac, were purchased from Gentest. The substrates, 7-ethoxycoumarin, diclofenac, 4- nitrophenol, leukotriene $B_4$ ($LTB_4$), along with the reaction products, 7-hydroxycoumarin, 4-nitrocatechol, 20-hydroxy-leukotriene $B_4$ (20-hydroxy-$LTB_4$), were purchased from Sigma, as were dicumarol, arylsulfatase, and β-glucuronidase. To assess the metabolic activity of different P450s in monolayer cell cultures, established methods were used (32). Briefly, both the tongue and liver cells cultured in 75 $cm^2$ flasks were treated with GTE (100 µg/ml) for 6 h or until the cells reached 80% confluency. Then the medium containing GTE was removed and the cells were washed with PBS to eliminate the residual GTE. The assay was started by addition of new media and specific substrate for different CYP isoforms. In addition to the cells without GTE treatment, two more control groups were also included in the experiment. Control groups included (1) cultures in which growth medium and substrate were present but no cells, and (2) cultures where medium and cells were present but no substrate. These cultures served as a negative control for the product formation. After overnight

incubation, the medium was collected and the metabolite produced by the cells in the medium was measured using either HPLC for CYPs 2C9, 2E1, and 4F3 or a fluorescence reader for CYP1A1/1A2 and CYP2D6.

To measure CYP1A1/1A2 and CYP 2D6 activities, the cultures incubated with 10 µM 7-ethoxycoumarin and 25 µM MAMC were exposed to 10 and 25 µM dicumarol, respectively, to inhibit conjugation of the dealkylated product (Gomez-Lechon et al., 1997; Onderwater et al., 1999). The respective 7-ethoxycoumarin O-deethlyase and MAMC O-demethylase activities were measured as described (Onderwater et al., 1999; Raner et al., 2002). Samples from the incubations with 250 µM diclofenac, 250 µM 4-nitrophenol, and 100 µM leukotriene $B_4$ (Christmas et al., 2001) were incubated with 1200 Roy units/ml of arylsulfatase and 200 Fishman units/ml of β-glucuronidase for 2 h at 37 °C before the metabolism analysis (Gomez-Lechon et al., 1997) to hydrolyze the conjugated products, if any. The substrates used to probe different CYP isoforms, the substrate concentrations, and the respective product are listed in Table 2.

### *Statistical analysis of data.*

The statistical analysis of the staining intensities by RT-PCR and immunoblot was determined by the Student's t test using the statistical analysis program in Microsoft Excel 97. Differences were considered significant when $P < 0.05$. All values reported are means ± SD for a minimum of three values.

## RESULTS

### *Constitutive expression of P450 isoforms in CAL 27 and HepG2 cells*

A total of 10 different human P450 isoforms (1A1, 1A2, 2A6, 2B6/7, 2C, 2D6, 2E1, 3A3/4, 3A7, and 4F3) were probed in both the human tongue and liver cells using RTPCR. Expression of 1A1, 1A2, 2C, 2D6, 2E1, and 4F3 was observed in tongue cells. The HepG2 liver cells expressed the same P450s as the CAL 27 tongue cells and also CYP2B6/7 and 3A7. Isoforms 2A6 and 3A3/4 were not detected in either cell types (data not shown).

### *Induction of CYP1A1 and 1A2 isoforms by 0-napthoflavone and CYP2C by all-trans retinoic acid and farnesol*

As shown in Fig. 1, the levels of CYP1A1 and CYP1A2 m RNA were significantly increased in tongue and liver cells after incubation with 100 µM β-napthoflavone. However, no further induction was observed beyond 1 h in either cell line. In addition to xenobiotic inducers such as β-napthoflavone, several endogenous compounds were tested as inducers of P450 expression. Fig. 2 shows that the expression of CYP2C was weakly induced in tongue and liver cells after 1 h treatment with 20 µM all-trans retinoic acid and 80 µM farnesol. Once again, no induction beyond 1 h was observed.

Table 2
Cytochrome *P*450 substrates, expected reaction products, and substrate concentrations selected for use in catalytic assays

| CYP isoform | Substrate | Substrate concentration (µM) | Product |
|---|---|---|---|
| 1A1/1A2 | 7-Ethoxycoumarin | 10 | 7-Hydroxycoumarin |
| 2C | Diclofenac | 250 | 4-Hydroxydiclofenac |
| 2D6 | MAMC[a] | 25 | HAMC[b] |
| 2E1 | 4-Nitrophenol | 250 | 4-Nitrocatechol |
| 4F3 | Leukotriene $B_4$ | 100 | 20-Hydroxy-$LTB_4$[c] |

[a] MAMC, 7-Methoxy-4-(aminomethyl)-coumarin.
[b] HAMC, 7-Hydroxy-4-(aminomethyl)-coumarin.
[c] 20-Hydroxy-$LTB_4$, 20-Hydroxy-Leukotriene $B_4$.

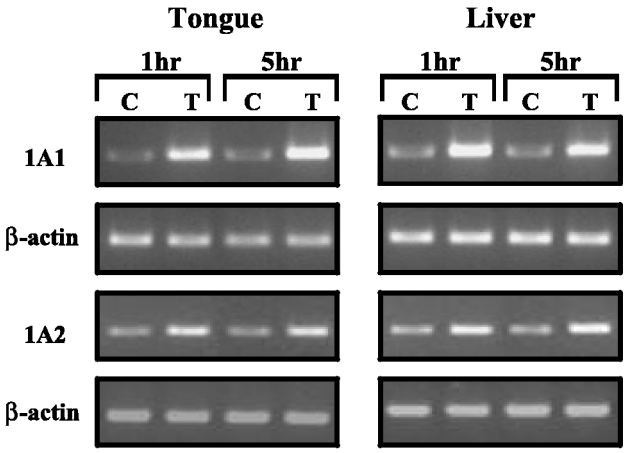


Fig. 1. RT-PCR analysis of CYP1A1 and 1A2 expression in tongue (CAL 27) and liver cells (HepG2) after being induced by 100 μM β-napthoflavone for 1 and 5 h. C, control cells; T, β-napthoflavone-treated cells.

### *Western blot analysis of CYP1A induction by β-napthoflavone*

Because expression of the CYP1A mRNA was induced by β-napthoflavone, a Western blot analysis was conducted to determine whether levels of mRNA would correlate with levels of protein. As indicated in Fig. 3, 100 μM pnapthoflavone caused a significant increase in the amount of CYP1A protein in tongue and liver cells. The two different induction periods, 1 and 5 h, produced similar effects in the two cell lines.

### *The effects of green tea extract on the expression of various P450 isoforms in CAL 27 and HepG2 cells*

Because the mRNA transcripts for CYPs 1A1, 1A2, 2C, 2D6, 2E1, and 4F3 were found to be expressed in both the tongue and liver cells, the effects of GTE on these isoforms were examined. RT-PCR analysis demon-

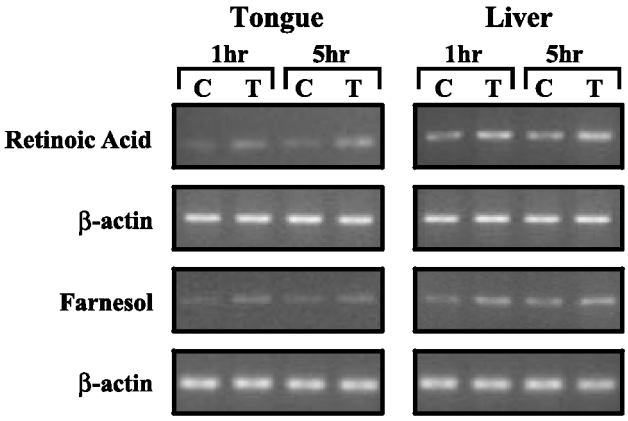


Fig. 2. RT-PCR analysis of CYP2C expression in tongue (CAL 27) and liver cells (HepG2) after being induced by 20 μM retinoic acid and 80 μM farnesol for 1 and 5 h. C, control cells; T, inducer (retinoic acid or farnesol)-treated cells.

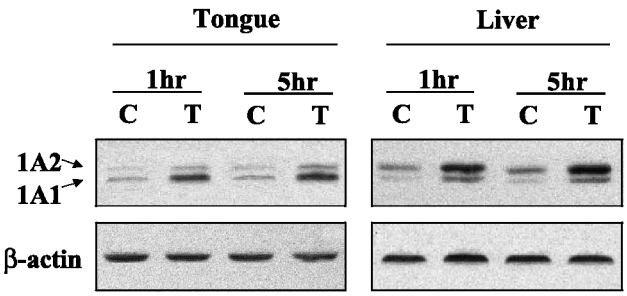


Fig. 3. Western blot analysis of the expression of CYP1A protein in tongue (CAL 27) and liver cells (HepG2) after being induced by 100 μM β-napthoflavone for 1 and 5 h, and stained with the goat anti-rat CYP1A antibody. C, control cells; T, β-napthoflavone-treated cells.

strated that GTE treatment for 6 h had an inductive effect on the expression of all the isoforms examined in tongue cells with the exception of CYP4F3 (Fig. 4). The effect on CYPs 1A1, 1A2, 2C, and 2E1 was dependent on GTE concentration. The induction of CYP2D6 by GTE treatment in the two cell types was mild, yet significant compared to the control. Three independent experiments yielded nearly identical results. Analysis of liver cells showed a similar induction pattern for these isoforms after GTE treatment (Fig. 4). The four catechins EGC, EGCG, EG, and ECG were quantified in the GTE extract used in this study. Using standard curves for each of the individual catechins, it was determined that catechins in the final 10.0-ml cultures containing 100 μl GTE were present at concentrations of 24.0 μg/ml for EGCG, 3.4 μg/ ml for EC, 19.2 μg/ml for EGC, and 7.9 μg/ml for ECG. Both HepG2 and Cal-27 cell cultures were treated with each of the catechin components at the concentrations listed above and RT-PCR analysis was performed to examine the effects of the individual catechins on CYP1A1 and CYP1A2 expression. None of the catechins alone produced an inductive effect on these isoforms (data not shown).

### *P450-dependent metabolism in CAL 27 and HepG2 cells*

Based on results from RT-PCR, GTE treatment at 100 μg/ml had the most significant effects on the CYPs examined. Therefore, 100 μg/ml of GTE was selected to treat the cells. Both tongue and liver cells showed significant 7-ethoxycoumarin deethylase (ECOD) activity based on overnight incubation with the substrate with or without 6 h GTE treatment (Fig. 5). GTE treatment had a significant inhibitory effect on ECOD activity in the two cell types. The activity decreased 64% and 61% in the tongue and liver cells, respectively. Metabolism of the other substrates was absent or minute in the two cell types. The diclofenac and p-nitrophenol hydroxylase activities were confirmed in the cell cultures using authentic product standards; however, the activities were near the detection limit. Consequently, the inhibitory effect of GTE could not be determined conclusively.

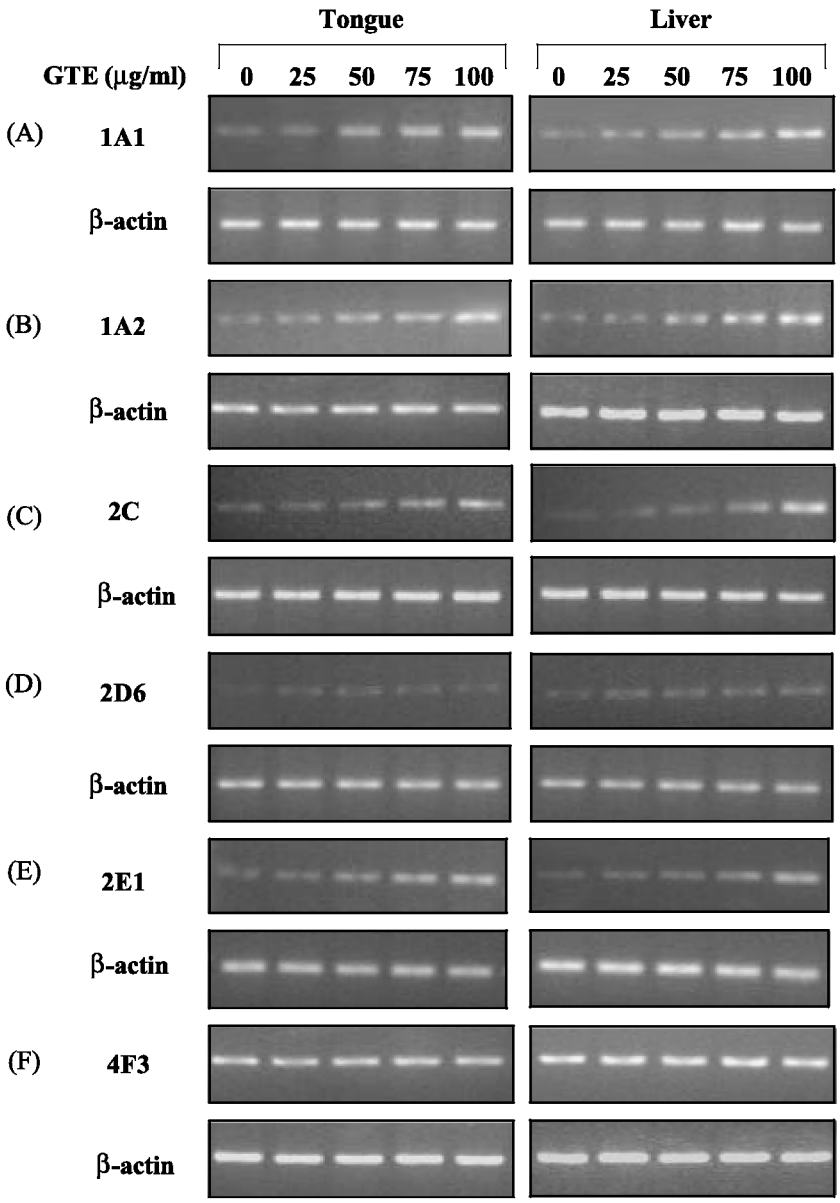


Fig. 4. The effects of GTE at different concentrations on the mRNA expression of (A) CYP1A1, (B) CYP1A2, (C) CYP2C, (D) CYP2D6, (E) CYP2E1, and (F) CYP4F3. The cells cultured in 25 $cm^2$ flasks were treated with 25, 50, 75, and 100 μg/ml of GTE for 6 h, followed by total RNA extraction and reverse transcription of 10 μg of RNA. The PCR products were amplified from 5 μg of the cDNA after 30 cycles. PCR products were recorded after gel electrophoresis and staining with ethidium bromide. Tongue, CAL 27 cells; Liver, HepG2 cells.

### *Western blot analysis of CYP1A and CYP2E1 by green tea extract*

Western blot analysis was used to determine whether the inhibitory effects of GTE on ECOD activity were due to a decrease in protein amount. In addition, because CYP2E1 protein has been known to be degraded rapidly in many tissue types, this isoform was included to examine whether the protein was expressed in tongue cells. In accordance with RT-PCR results, 100 μg/ml of GTE treatment for 6 h caused a slight increase in the amount of CYP1A protein in tongue and liver cells (Fig. 6). However, both cell types contained no detectable level of CYP2E1 protein by Western blot analysis with or without GTE treatment.

## DISCUSSION

The extrahepatic expression of P450 enzymes in humans is a growing area of interest; however, the oral cavity remains poorly characterized with respect to its ability to interact with foreign chemicals. In the current study, the expression of six different P450 isoforms (1A1, 1A2, 2C, 2D6, 2E1, and 4F3) in tongue cells was demonstrated by RT-PCR analysis. The results were in accordance with our

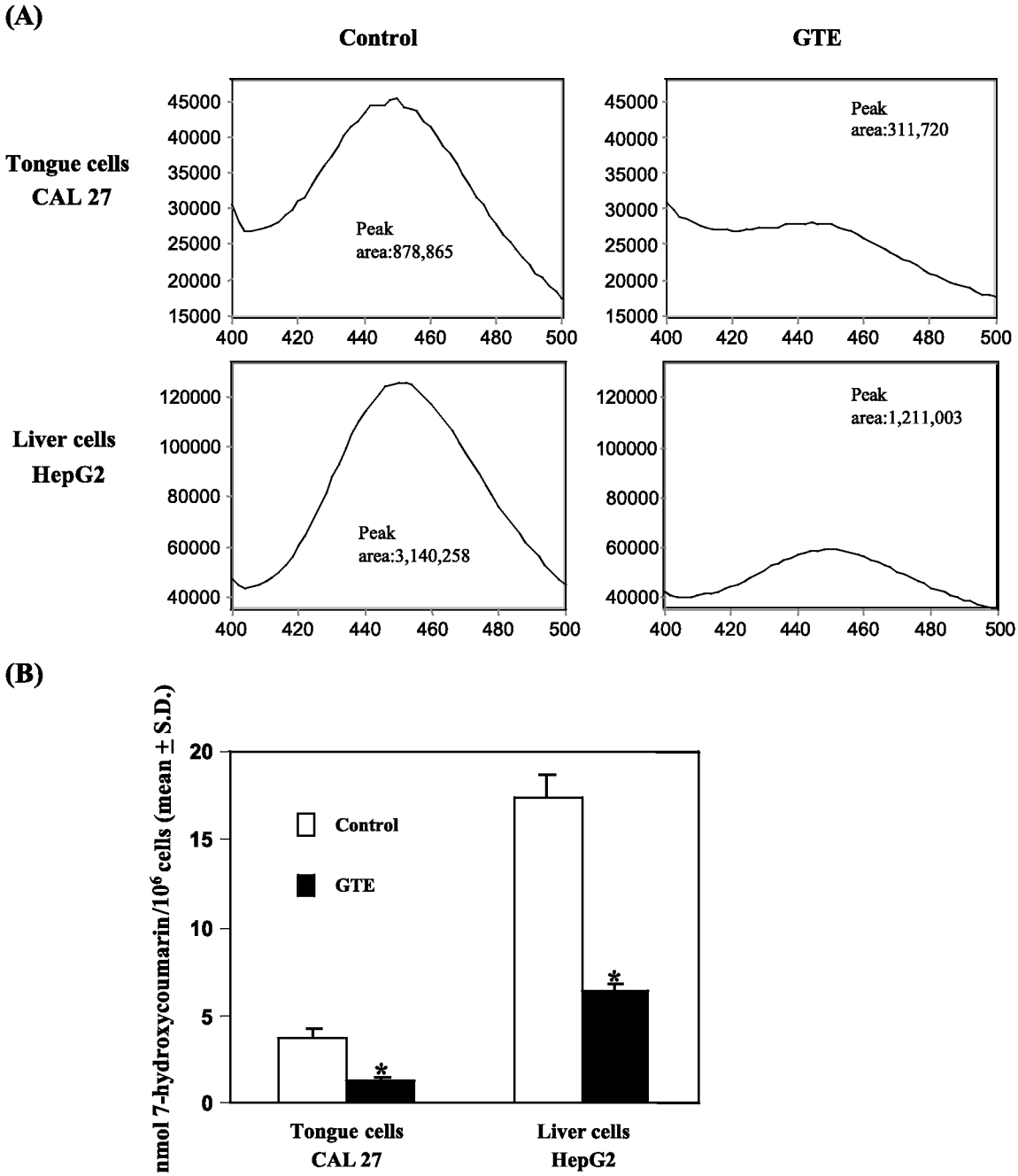


Fig. 5. The inhibitory effects of GTE at 100 μg/ml on ECOD activity. (A) Cells were treated with GTE for 6 h and ECOD activity was determined fluorometrically. Values present the averages from three independent experiments. (B) Bar graph showing the absolute activities with and without GTE treatment for 6 h. *Statistically significant (Student's $t$ test; $P < 0.05$).

previous findings that showed the expression of CYPs 1A1, 2E1, and 4A4 in rabbit tongue tissue (Yang et al., 2003). The presence of CYPs 1A, 2C, 2D6, and 2E1 in the oral cavity has been reported previously (Vondracek et al., 2001, 2002). However, the identification of CYP expression and inducibility in human tongue cells are novel findings.

CYP2D6 is absent or has low-level expression in the oral cavity (Vondracek et al., 2001), which is consistent with our observations that its expression is relatively weak compared with other isoforms in the tongue cell model. The results were negative for the expression of CYP3A in tongue cells, yet CYP3A4/7 and CYP3A5 have been detected by RTPCR in buccal cells (Vondracek et al., 2001). It has been reported previously that HepG2 cells do not express CYP3A4, rather the fetal isoform, CYP3A7 is expressed preferentially (Schuetz et al., 1993), which is consistent with our findings.

In a prior study, we observed the expression of CYP4A4 in rabbit tongue tissue based on RT-PCR data

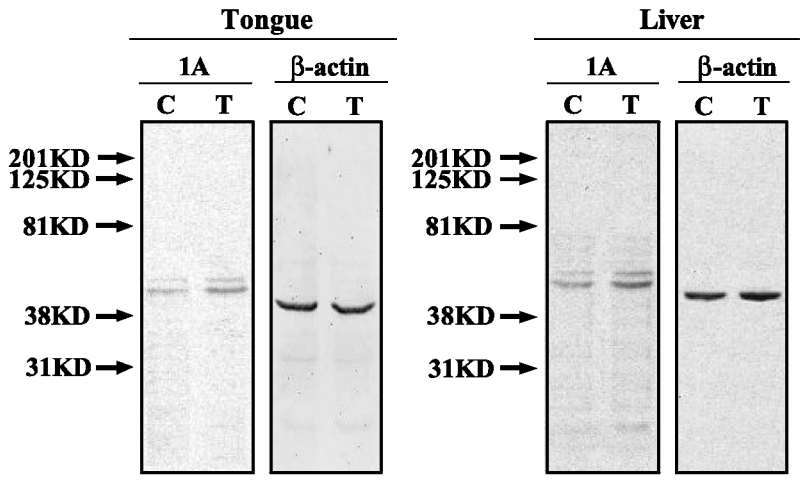


Fig. 6. Western blot analysis of the expression of CYP1A protein in tongue (CAL 27) and liver cells (HepG2) after treatment with 100 μg/ml of GTE for 6 h and stained with the goat anti-rat CYP1A1 antibody. The bands were compared to pre-stained protein standards. C, control cells; T, GTE-treated cells.

(Yang et al., 2003). In the present study, the expression of CYP4F3, another member in the CYP4 family, was found in human tongue cells. CYP4 enzymes including CYP4A and CYP4F catalyze ù-hydroxylation of fatty acids (Lasker et al., 2000). CYP4F gene products have been shown to be expressed in the liver and many other tissue types, such as kidney, prostate, blood leukocyte, and bone marrow (Christmas et al., 2001).

Although RT-PCR data indicated the expression of CYP2E1 in tongue and liver cells, Western blotting analysis failed to detect 2E1 protein. It has been reported that the CYP2E1 protein is rapidly degraded in many tissue types (Zhukov and Ingelman-Sundberg, 1999), and the literature suggests that in rat tongue, the 2E1 protein is not stable unless the stabilizer ethanol is present. That 2E1 mRNA is produced in the human tongue may have significant implications for understanding the mechanism of ethanol-induced oral carcinogenesis.

The induction of specific isoforms in tongue cells was demonstrated using β-napthoflavone, all-trans retinoic acid, and farnesol, and GTE at different concentrations. RT-PCR and Western blot analysis indicated that both mRNA and protein levels of CYP1A enzymes in tongue cells increased significantly after β-napthoflavone treatment, and with GTE treatment to a lesser extent. Several studies have shown that β-napthoflavone induces liver CYP1A mRNA and its related activity (Ghosh et al., 2001). However, previous studies using HepG2 cells indicated a lack of correlation between mRNA levels and protein levels for CYP1A2 (Li et al., 1998; Vakharia et al., 2001). More specifically, the 1A2 isoform was detected by RT-PCR, but not detected by Western blotting analysis in the HepG2 cell line. More recently, Chan et al. (2003) and Bacon et al. (2003) have reported basil levels of CYP1A2 activities in HepG2 cells, and Feng et al. (2003) have presented immunoblotting data consistent with low levels of 1A2 protein being present in HepG2 cells. Furthermore, in the current study, microsomes were used in the immunoblotting study, which are more highly enriched in P450 enzymes than the S9 fractions used previously, and 100 μg of total microsomal protein was loaded into each well compared with 25 μg of the S9 samples used. The data presented here support the view that both P4501A1 and 1A2 protein are expressed in HepG2 and Cal-27 cells and that expression is induced in both cell types by β-napthoflavone and, to a lesser extent, GTE. It has been reported that treatment with green tea results in accumulation of CYP1A1 mRNA and protein in HepG2 cells (Williams et al., 2000), presumably via an AhR-dependent process (Denison and Whitlock, 1995). Green tea extracts were suggested to contain low levels of AhR agonists or low-affinity AhR agonists (Williams et al., 2000). Our results indicate AhR and other regulator proteins involved in PAH induction of P450s appear to be present in CAL 27 cells.

The expression of CYP2C was increased in the two cell types by all-trans retinoic acid, farnesol, and GTE in a dose-dependent manner. CYP2C is known to be regulated by retinoic acid through nuclear receptors such as

RAR and RXR (Honkakoski and Negishi, 2000). Farnesol is a terpenoid compound produced endogenously that has been shown to activate the farnesoid X receptor in vivo (Forman et al., 1995) and a recent report suggests that farnesol may inhibit CYP2C enzymes in rabbit liver (Raner et al., 2002). The induction of 2C by farnesol in HepG2 and Cal 27 cells, although very weak in nature, is the first such report and may indicate a possible regulation of CYP2C enzymes through the FXR/RXR heterodimer.

With the exception of CYP1A, the effect of GTE on gene expression of other CYPs examined in this study has never been reported. RT-PCR analysis showed that both CYP2C and CYP2E1 were induced in two cell types by GTE treatment. CYP2D6 was also slightly induced by GTE, whereas the induction was not concentration dependent. Of all the isoforms examined in the study, CYP4F3 was the only isoform that was not affected by GTE. The similar induction patterns between CAL 27 and HepG2 cells suggest that the regulation of P450 isoforms by GTE in the human tongue may be similar to that in the human liver. It can therefore be concluded that the regulation of CYP gene expression by GTE does not involve liver-specific factors. Furthermore, none of the four major tea catechins produced this effect in HepG2 or Cal-27 cells. This suggests that the observed induction is not the result of hydrogen peroxide formation that occurs via auto-oxidation of the catechins. Chen et al. (1996) have shown that in rats, induction of CYP 1A2 by tea is related to caffeine content and not catechins, which is consistent with the results presented here.

Induction of CYP2E1 by ethanol has been studied extensively and is believed to occur largely through protein stabilization (Yang et al., 1990). Recently, CYP2E1 was shown to be regulated at the transcriptional level by a variety of other compounds including procymidone (Sapone et al., 2003), DMSO (Chauret et al., 1998), and phorbol esters (Tindberg, 2003). Although a specific mechanism has not been identified, several groups have shown that 2E1 can be transcriptionally regulated by cytokines, possibly via the transcription activator SP1 (Peng and Coon, 2000). Regulation of CYP2C enzymes has been described in terms of activation of nuclear receptors PXR, VDR, and GR (Pascussi et al., 2003); however because CYP2E1 is not a target of any of these receptors, it is unlikely that GTE exerts its effects through these receptors. In fact, the broad spectrum of CYPs induced by GTE suggests that either the induction is occurring via a global regulatory pathway or there are multiple components in GTE exerting P450-inductive effects.

The effects on CYP1A1 and CYP1A2 activities by green tea have been studied in animal models and some cell-based systems. At low concentrations, GTE has an apparent stimulatory effect on P450 activity (Maliakal et al., 2001), whereas higher doses result in inhibition (Bu-Abbas et al., 1994). Our results demonstrated that although mRNA and protein levels of CYP1A were increased in response to GTE treatment of both CAL-27 and HepG2 cells, CYP1A-related ECOD activities were markedly inhibited after treatment with GTE (Fig. 5). The discrepancy could be attributed to the weak agonist activity of green tea, which may slightly increase CYP1A enzyme levels, while compounds present in the extract inactivate the enzyme that is produced. The net result would be more enzyme but less activity. Tea contains high levels of catechins that have previously been shown to inhibit the NADPH-dependent reduction of CYP (Steele et al., 1985), and recent studies have shown that green tea and its polyphenols inhibit NADPH-cytochrome P450 reductase (OR) and further interfere with the electron flow to CYPs (Xu and Dashwood, 1999). It is worth noting that substrates were applied to the cells after removal of the media containing GTE. Thus, it is possible that GTE may cause an irreversible inhibitory effect on the or the reductase or CYP enzymes present. To our knowledge, this is the first report showing that GTE decreases certain CYP activities in oral tissue. Moreover, a recent study involving human subjects has found that holding a tea solution in the mouth for a few minutes without swallowing produced even higher salivary catechin levels than that observed after drinking tea (Yang et al., 1999). This observation implies the absorption of tea catechins through the oral mucosa and supports the possibility that tea catechins can be absorbed in the oral cavity. Along with our findings, these data support the concept that tea consumption exerts direct modulation effects on oral cytochrome P450s.

Other specific CYP substrates were used for the assessment of metabolic activity of other isoforms examined in the study, namely CYPs 2C9, 2E1, 2D6, and 4F3. However, the activities were found to be absent or very low, with or without GTE treatment, as indicated by the lack of metabolism of the corresponding substrates. It may

indicate low amounts of some mRNA or selective formation or degradation of the corresponding proteins in the oral cavity. The results were similar to a previous study regarding the activity of different CYPs in human buccal tissue (Vondracek et al., 2001).

In summary, our results demonstrate the constitutive and chemically induced expression of a variety of CYP genes in a cultured human tongue cancer cell line. Moreover, green tea consumption appears to impact xenobiotic metabolism in these cells. Further, the parallel assessment of tongue and liver cells reveals that CYP inductions in tongue cells are comparable to those in liver cells, which indicates that the induction of specific isoforms in the tongue cell line may occur through biochemical mechanisms that are analogous to those that are operative in the liver.

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